



ELISA PRODUCT INFORMATION & MANUAL

Human Lymphotoxin-alpha/TNF- beta ELISA Kit (Colorimetric)

NBP1-83730

Enzyme-linked Immunosorbent Assay for quantitative
detection. For research use only.

Not for diagnostic or therapeutic procedures.

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TABLE OF CONTENTS

1	Intended Use	3
2	Summary	3
3	Principles of the Test	5
4	Reagents Provided	6
5	Storage Instructions – ELISA Kit	8
6	Specimen Collection and Storage Instructions	8
7	Materials Required But Not Provided	9
8	Precautions for Use	10
9	Preparation of Reagents	12
10	Test Protocol	15
11	Calculation of Results	20
12	Limitations	23
13	Performance Characteristics	24
14	Reagent Preparation Summary	27
15	Test Protocol Summary	28

1 Intended Use

The human Lymphotoxin-alpha/TNF-beta ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Lymphotoxin-alpha/TNF-beta. **The human Lymphotoxin-alpha/TNF-beta ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2 Summary

Tumor Necrosis Factor (TNF) was originally discovered as a serum protein with necrotizing effects on certain transplantable mouse tumors in vivo and cytotoxic effects against some transformed cells in vitro. The TNF family consists of two proteins designated TNF alpha, also called cachectin, and TNF beta, also called lymphotoxin, which are pleiotropic cytokines that can mediate a wide variety of biological effects. TNF beta is produced by activated lymphocytes, whereas TNF alpha is mostly produced by activated macrophages. Soluble TNF beta is a T-cell derived glycoprotein of 25 kD encoded by a gene within the MHC. The molecule consists of a 17.5 to 18 kD polypeptide core and 7kD of N-linked carbohydrate. TNF beta is 28 % structurally homologous with the macrophage produced non-glycoprotein TNF alpha. The genes for TNF alpha and TNF beta are closely linked, and the proteins share biological activities. TNF has been shown to interact with a cell through specific high-affinity receptors with a few hundred up to more than 20.000 copies per cell. In a variety of cell lines, two different TNF-receptor proteins have been identified and the cDNAs cloned. Recently, an ELISA has been established by Adolf and Apfler which provides a simple, rapid, and highly sensitive method for the determination of soluble TNF-R (60 kDa) levels in body fluids or cell culture supernatants. TNF beta is induced in an antigen-specific MHC restricted fashion from class I and class II restricted T cells. Viral infection is also associated with TNF beta production by lymphoid cells. TNF beta has several effects on target cells including killing, growth stimulation, induction of adhesion molecule (ICAM-1) expression, and induction of differentiation. The mechanisms of TNF beta effects involve receptor binding and internalization and several sequelae including changes in prostaglandins and chromosome integrity. TNF beta participates in tumor immunity, and it has been reported to inhibit carcinogenesis as well as growth of some tumors in vivo.

Recent studies have demonstrated that both TNF alpha and TNF beta are capable of activating neutrophils in vitro. The exposure of neutrophils to TNF alpha or TNF beta causes the production of superoxide radicals, induces phagocytic response and enhances antibody dependent cell cytotoxicity. The release of IL-1 from human endothelial cells is also induced by TNF alpha and TNF beta. All the in vitro studies suggest that TNF beta may play an important role in immunoregulation. In fibroblasts TNF beta induces the synthesis of colony-stimulating factors, IL-1, collagenase and prostaglandin-E2. Monocytes are stimulated for terminal differentiation. On B-cells TNF beta acts as mitogen. As TNF beta exerts proliferative capacity on fibroblasts it may participate in the process of wound healing. Recently, TNF beta was found to belong to the group of endogenous pyrogens/sleep factors. Elevated TNF beta levels were also found in patients with adult T-cell leukemia and hypercalcemia, diabetes as well as malaria.

While both TNFs inhibit growth of tumor cells, they stimulate the growth of human lung fibroblasts and dermal fibroblasts. TNF beta also acts as antiviral agent against a variety of RNA (VSV and EMCV) and DNA (Ad-2 and HSV-2) viruses and its activity is potentiated by interferon-gamma in a synergistic fashion. Moreover, TNF beta contributes to the defense against parasitic infections and induces osteoclastic bone resorption and inhibition of bone collagen synthesis.

The elucidation of the physiological and pathophysiological role was limited due to a lack of adequate assay systems. The present assay developed by Adolf and Lamche provides a simple and rapid method for determination of serum levels of TNF beta with a minimal detectable dose as low as 7 pg/ml serum. This assay will help to clarify the possible diagnostic and prognostic value of circulating TNF beta in various neoplastic and inflammatory diseases. The assay detects recombinant, unglycosylated lymphotoxin with the same sensitivity as the natural, glycosylated protein, shows good correlation with the standard cytotoxicity bioassay, and is specific for biologically active TNF beta without TNF alpha cross reactivity.

3 Principles of the Test

An anti-human Lymphotoxin-alpha/TNF-beta coating antibody is adsorbed onto microwells.

Human Lymphotoxin-alpha/TNF-beta present in the sample or standard binds to antibodies adsorbed to the microwells and the HRP-conjugated anti-human Lymphotoxin-alpha/TNF-beta antibody is added and binds to human Lymphotoxin-alpha/TNF-beta captured by the first antibody.

Following incubation unbound HRP-conjugated anti-human Lymphotoxin-alpha/TNF-beta is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of human Lymphotoxin-alpha/TNF-beta present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human Lymphotoxin-alpha/TNF-beta standard dilutions and human Lymphotoxin-alpha/TNF-beta concentration determined.

Figure 1

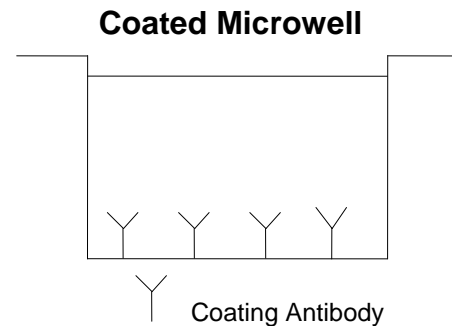


Figure 2

First Incubation

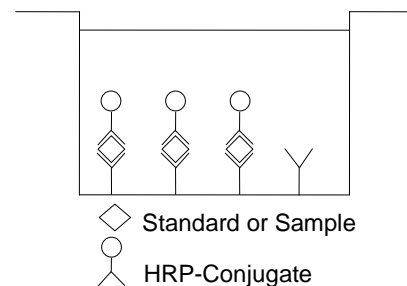


Figure 3

Second Incubation

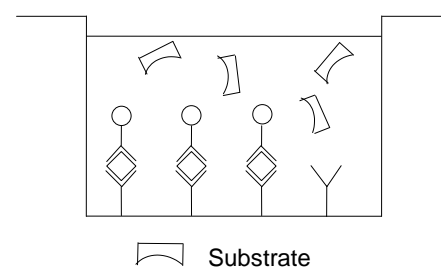
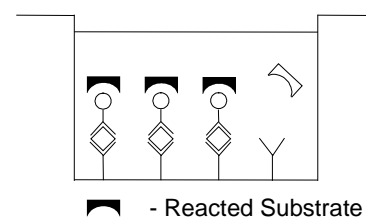


Figure 4



4 Reagents Provided

4.1 Reagents for human Lymphotoxin-alpha/TNF-beta ELISA NBP1-83730 (96 tests)

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human Lymphotoxin-alpha/TNF-beta
- 1 vial (200 µl) **HRP-Conjugate** anti-human Lymphotoxin-alpha/TNF-beta monoclonal antibody
- 2 vials (50 µl) human Lymphotoxin-alpha/TNF-beta **Standard** concentrate, 1 ng/ml upon dilution
- 1 vial (5 ml) **Sample Diluent**
- 1 vial (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
- 2 **Adhesive Films**

4.2 Reagents for human Lymphotoxin-alpha/TNF-beta ELISA NBP1-83730

(10x96 tests)

10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to human Lymphotoxin-alpha/TNF-beta

10 vials (200 µl) **HRP-Conjugate** anti-human Lymphotoxin-alpha/TNF-beta monoclonal antibody

10 vials (50 µl) human Lymphotoxin-alpha/TNF-beta **Standard** concentrate, 1 ng/ml upon dilution

5 vials (5 ml) **Sample Diluent**

1 vials (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)

2 bottles (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)

10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (100 ml) **Stop Solution** (1M Phosphoric acid)

10 **Adhesive Films**

5 Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels. Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6 Specimen Collection and Storage Instructions

Cell culture supernatant, serum and plasma were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Lymphotoxin-alpha/TNF-beta. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.4).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 μ l to 1000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

8 Precautions for Use

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9 Preparation of Reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.

If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water.

Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 HRP-Conjugate

Please note that the HRP-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **HRP-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

9.4 Human Lymphotoxin-alpha/TNF-beta Standard

Prepare **human Lymphotoxin-alpha/TNF-beta standard** by addition of Sample Diluent or another appropriate diluent * as stated on the label and mix gently (concentration of standard = 1 ng/ml).

After usage remaining standard cannot be stored and has to be discarded.

(The highest standard concentration for the standard curve, 1 ng/ml is named S1.)

* Use Sample Diluent for serum and plasma samples and another appropriate diluent for cell culture samples, e.g. cell culture medium.

Standard dilutions can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.4.1).

9.4.1 External Standard Dilution

Label 7 tubes, one for each standard point.

S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:

Pipette 225 μ l of Sample Diluent * into tubes S2 – S7.

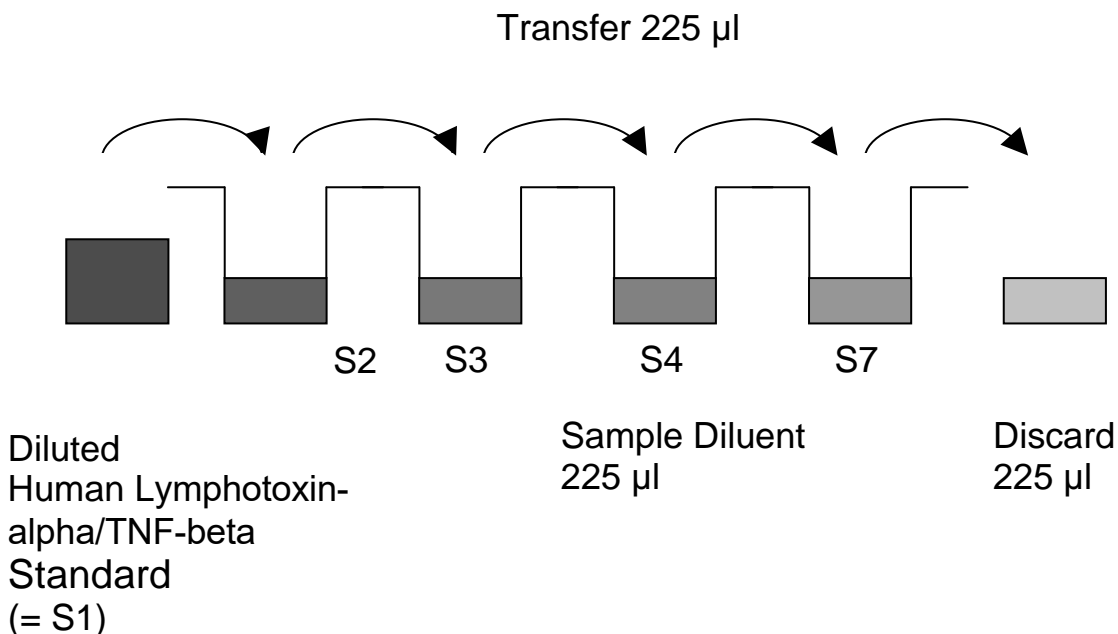
Pipette 225 μ l of diluted standard (serves as the highest standard S1, concentration of standard 1 = 1000 pg/ml) into the first tube, labelled S2, and mix (concentration of standard 2 = 500 pg/ml).

Pipette 225 μ l of this dilution into the second tube, labelled S3, and mix thoroughly before the next transfer.

Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 5).

Sample Diluent * serves as blank.

Figure 5



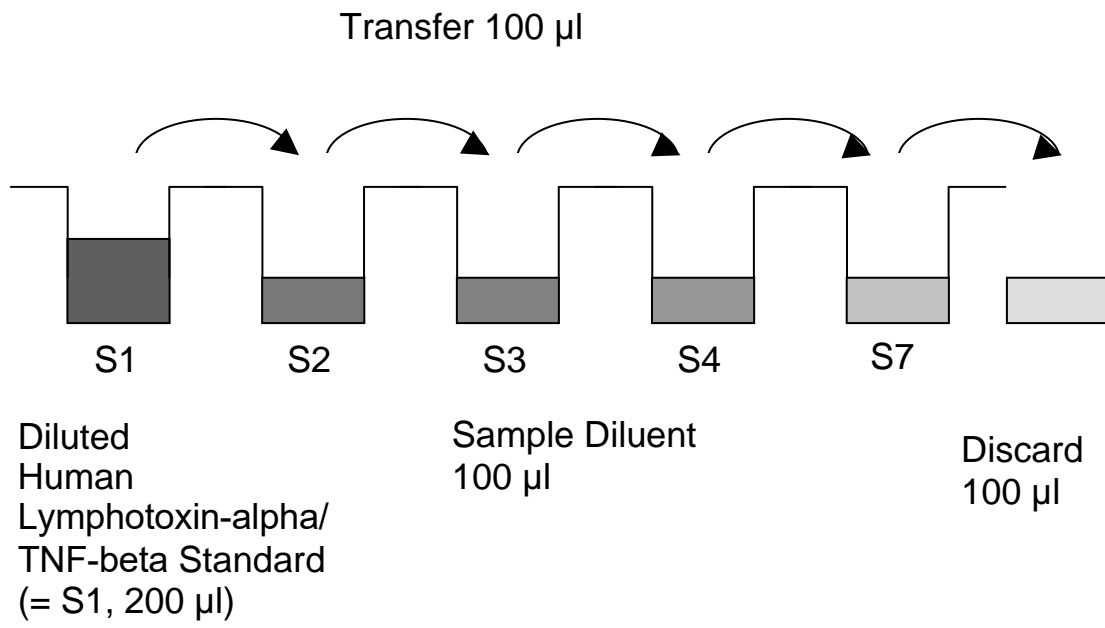
* Use Sample Diluent for serum and plasma samples and another appropriate diluent for cell culture samples, e.g. cell culture medium.

10 Test Protocol

- a. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- b. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**
- c. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.4.1):
Add 100 µl of Sample Diluent * in duplicate to **standard wells** B1/2-G1/2, leaving A1/A2 empty. Pipette 200 µl of prepared **standard** (see Preparation of Standard 9.4, concentration = 1000.0 pg/ml) in duplicate into well A1 and A2 (see Table 1) and transfer 100 µl to wells B1 and B2. Mix the contents of wells B1 and B2 by repeated aspiration and ejection, and transfer 100 µl to wells C1 and C2, respectively. (see Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human Lymphotoxin-alpha/TNF-beta standard dilutions ranging from 1000.0 to 15.6 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

* Use Sample Diluent for serum and plasma samples and another appropriate diluent for cell culture samples, e.g. cell culture medium.

Figure 6



In case of an **external standard dilution** (see 9.4.1), pipette 100 µl of these standard dilutions (S1 –S7) in the standard wells according to Table 1.

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (1000.0 pg/ml)	Standard 1 (1000.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (500.0 pg/ml)	Standard 2 (500.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (250.0 pg/ml)	Standard 3 (250.0 pg/ml)	Sample 3	Sample 3
D	Standard 4 (125.0 pg/ml)	Standard 4 (125.0 pg/ml)	Sample 4	Sample 4
E	Standard 5 (62.5 pg/ml)	Standard 5 (62.5 pg/ml)	Sample 5	Sample 5
F	Standard 6 (31.3 pg/ml)	Standard 6 (31.3 pg/ml)	Sample 6	Sample 6
G	Standard 7 (15.6 pg/ml)	Standard 7 (15.6 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- d. Add 100 µl of **Sample Diluent** *in duplicate to the **blank wells**.
- e. Add 100 µl of each **sample** in duplicate to the **sample wells**.
- f. Prepare **HRP-Conjugate** (see Preparation of HRP-Conjugate 9.3).
- g. Add 50 µl of **HRP-Conjugate** to all wells.
- h. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 4 hours, if available on a microplate shaker set at 400 rpm.
- i. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.
- j. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- k. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

- l. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

* Use Sample Diluent for serum and plasma samples and another appropriate diluent for cell culture samples, e.g. cell culture medium.

m. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human TNF beta concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human Lymphotoxin-alpha/TNF-beta for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human Lymphotoxin-alpha/TNF-beta concentration.
- **If instructions in this protocol have been followed, samples have not been diluted and the concentration read from the standard curve must not be multiplied by a dilution factor.**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Lymphotoxin-alpha/TNF-beta levels. Such samples require further external predilution according to expected human Lymphotoxin-alpha/TNF-beta values with an appropriate diluent in order to precisely quantitate the actual human Lymphotoxin-alpha/TNF-beta level.**
- It is suggested that each testing facility establishes a control sample of known human Lymphotoxin-alpha/TNF-beta concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7

Representative standard curve for human Lymphotoxin-alpha/TNF-beta ELISA. Human Lymphotoxin-alpha/TNF-beta was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

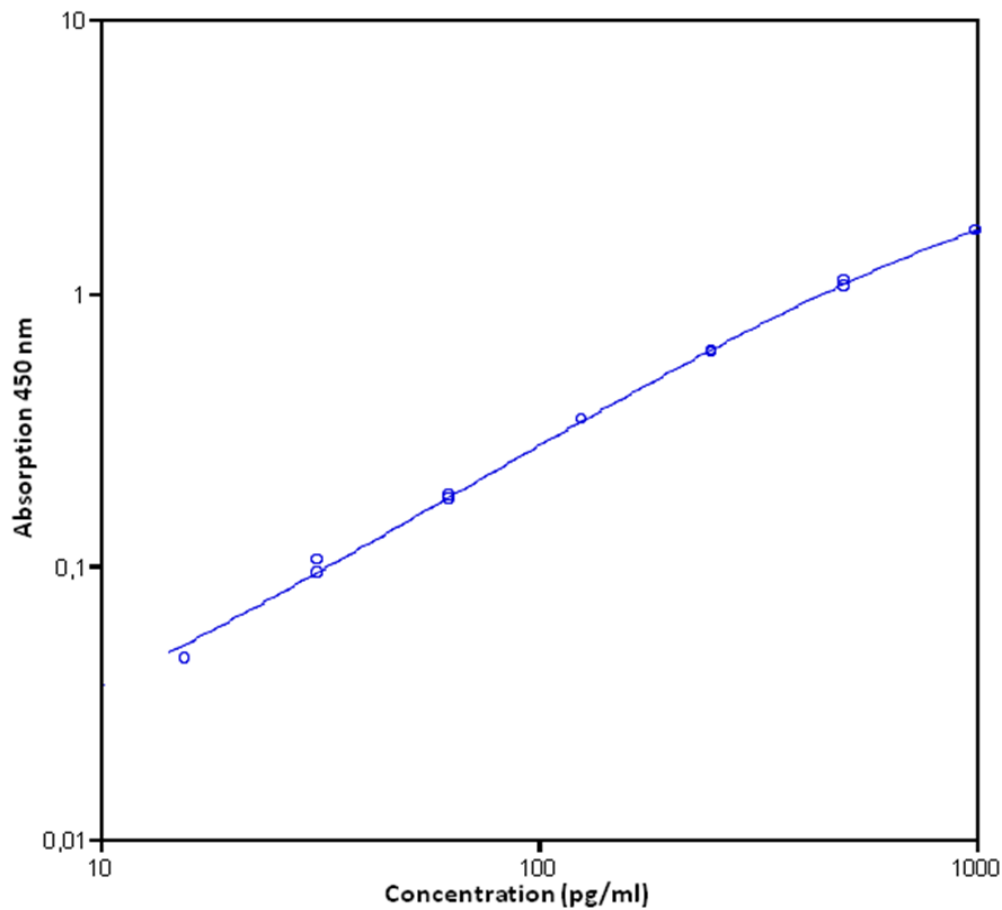


Table 2

Typical data using the human Lymphotoxin-alpha/TNF-beta ELISA
 Measuring wavelength: 450 nm
 Reference wavelength: 620 nm

Standard	Human Lymphotoxin-alpha/ TNF-beta Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	1000.0	1.687 1.700	1.693	0.5
2	500.0	1.112 1.061	1.086	3.3
3	250.0	0.605 0.619	0.612	1.6
4	125.0	0.345 0.344	0.344	0.2
5	62.5	0.183 0.175	0.179	3.2
6	31.3	0.105 0.095	0.100	7.1
7	15.6	0.046 0.046	0.046	0.0
Blank	0	0.014 0.015	0.015	3.4

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of human Lymphotoxin-alpha/TNF-beta defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 4.6 pg/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 5 independent experiments. Each assay was carried out with 4 replicates of 3 serum samples containing different concentrations of human Lymphotoxin-alpha/TNF-beta. 2 standard curves were run on each plate. Data below show the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 8.0%.

Table 3

	CV Sample 1 high (%)	CV Sample 2 medium (%)	CV Sample 3 low (%)	Mean intra- assay CV (%)
h Lymphotoxin- alpha/TNF-beta	8.5	8.2	7.3	8.0

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 4 replicates of 3 serum samples containing different concentrations of human TNF beta. 2 standard curves were run on each plate. Data below show the coefficient of variation calculated on 12 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 10.2%.

Table 4

	CV Sample 1 high (%)	CV Sample 2 medium (%)	CV Sample 3 low (%)	Mean inter- assay CV (%)
h Lymphotoxin- alpha/TNF-beta	10.6	11.2	8.7	10.2

13.3 Spike Recovery

The spike recovery was evaluated by spiking 2 levels of human Lymphotoxin-alpha/TNF-beta into 5 serum samples. Recoveries were determined in 3 independent experiments with 4 replicates each. The unspiked serum was used as blank in these experiments. The recovery ranged from 87% to 130% with an overall mean recovery of 105%.

13.4 Sample Stability

13.4.1 Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed 5 times, and the human Lymphotoxin-alpha/TNF-beta levels determined. There was no significant loss of human Lymphotoxin-alpha/TNF-beta concentrations between 0 and 5 freeze-thaw cycles.

13.4.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human Lymphotoxin-alpha/TNF-beta level determined after 24 h. There was no significant loss of human Lymphotoxin-alpha/TNF-beta immunoreactivity during storage under above conditions.

13.5 Specificity

The Lymphotoxin-alpha/TNF-beta ELISA is specific for human Lymphotoxin-alpha/TNF-beta. Only biologically active protein is recognized by the antibodies, since inactivation of Lymphotoxin-alpha/TNF-beta measured by bioassay results in a parallel decrease in immunoreactivity. Natural, glycosylated Lymphotoxin-alpha/TNF-beta shows the same reactivity as recombinant, unglycosylated protein. The test is unaffected by the presence of denatured Lymphotoxin-alpha/TNF-beta, recombinant human TNF alpha even at the highest concentration tested (0.5 mg/ml), or recombinant human interferon alpha 2c at a concentration up to 1 mg/ml.

Unexpectedly, recombinant human interferon gamma, which is unrelated to Lymphotoxin-alpha/TNF-beta in its primary structure, showed a very low but reproducible cross-reactivity of 0.00012 %. Since serum levels of interferon gamma are well below 1 ng/ml, this cross-reactivity is irrelevant for all practical purposes. There was no interference with TNF-R (60 kDa) up to a concentration of 400 ng/ml.

13.6 Expected Values

A panel of 40 sera samples from randomly selected apparently healthy donors (males and females) was tested for human Lymphotoxin-alpha/TNF-beta. The detected human TNF beta levels ranged between 1.0 and 1125.5 pg/ml with a mean level of 140.4 pg/ml and a standard deviation of 309 pg/ml.

The levels measured may vary with the sample collection used.

13.7 Calibration

The immunoassay is calibrated with highly purified recombinant human Lymphotoxin-alpha/TNF-beta which has been evaluated against the international Reference Standard NIBSC 87/640 and has been shown to be equivalent. NIBSC 87/640 is quantitated in International Units (IU), 1IU corresponding to 6.7 pg human TNF beta.

14 Reagent Preparation Summary

14.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20x (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

14.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

14.3 HRP-Conjugate

Make a 1:100 dilution of **HRP-Conjugate** in Assay Buffer (1x)

Number of Strips	HRP-Conjugate (ml)	Assay Buffer (1x)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

14.4 Human Lymphotoxin-alpha/TNF-beta Standard

Prepare **human Lymphotoxin-alpha/TNF-beta standard** by addition of Sample Diluent * as stated on the label and swirl or mix gently.

* Use Sample Diluent for serum and plasma samples and another appropriate diluent for cell culture samples, e.g. cell culture medium.

15 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent *, in duplicate, to standard wells leaving the first wells empty. Pipette 200 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
Alternatively external standard dilution in tubes (see 9.4.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent *, in duplicate, to the blank wells.
5. Add 100 µl sample in duplicate, to designated sample wells.
6. Prepare HRP-Conjugate.
7. Add 50 µl HRP-Conjugate to all wells.
8. Cover microwell strips and incubate 4 hours at room temperature (18° to 25°C).
9. Empty and wash microwell strips 3 times with Wash Buffer.
10. Add 100 µl of TMB Substrate Solution to all wells.
11. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
12. Add 100 µl Stop Solution to all wells.
13. Blank microwell reader and measure colour intensity at 450 nm.

* Use Sample Diluent for serum and plasma samples and another appropriate diluent for cell culture samples, e.g. cell culture medium.

Note: If instructions in this protocol have been followed, samples have not been diluted and the concentration read from the standard curve must not be multiplied by a dilution factor.